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(54) High density media for mammalian cell culture

(57) Mammalian cell culture media are prepared by balancedly fortifying the nutrient components of a conventional cell culture medium, which permit high-density culture of mammalian cells, in batch and fed-batch mode which enhances cell growth and product yield. The components added are preferably glycine and glutamine.

Fig 1

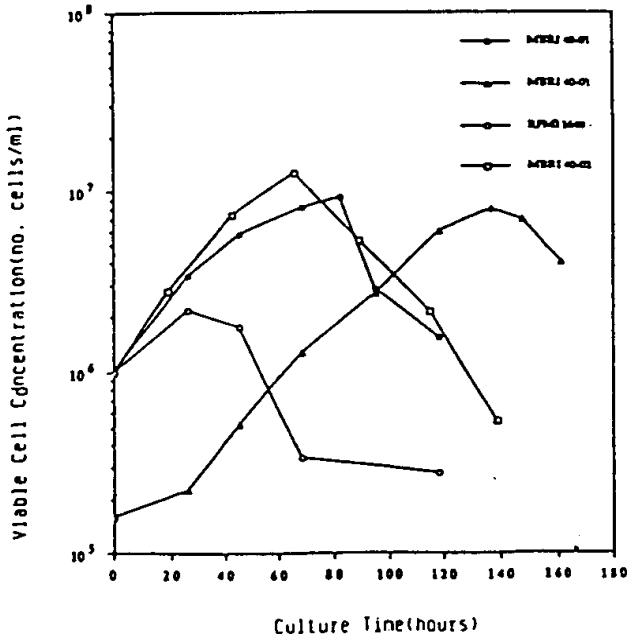

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Fig 1

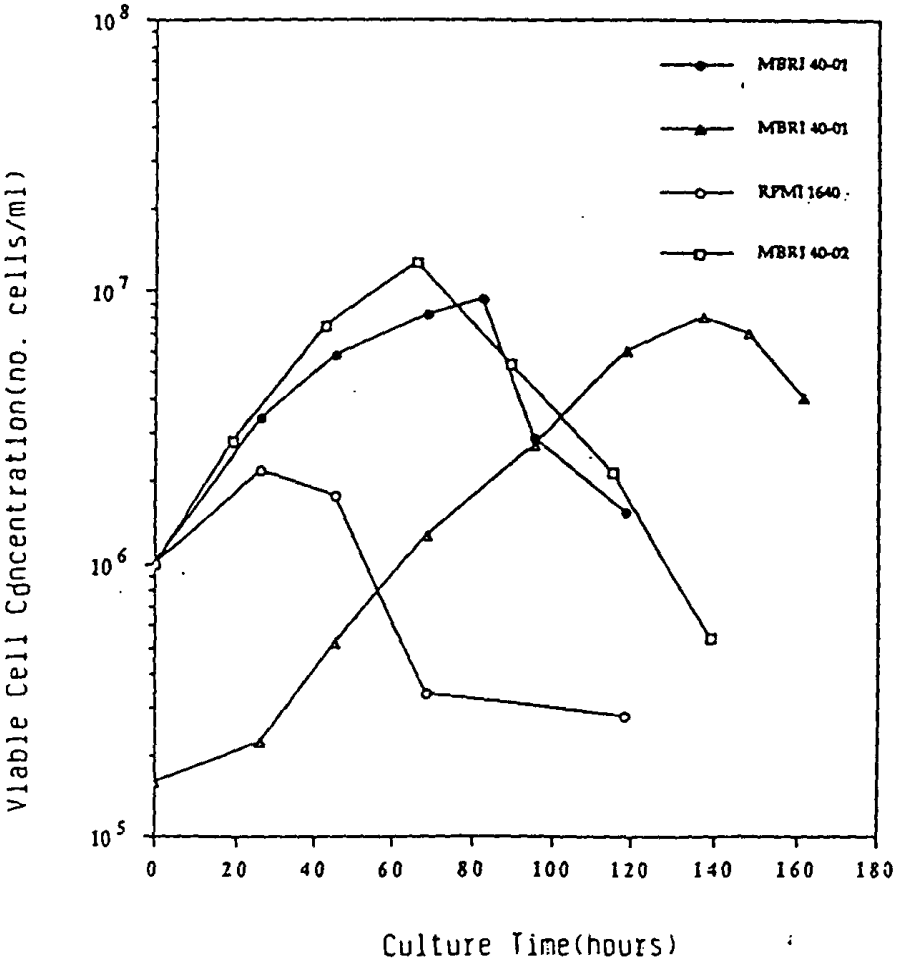

MBRI 40-01
MBRI 40-01
RPMI 1640
MBRI 40-02
Viable Cell Concentration(no. cells/ml)
10^8
10^7
10^6
10^5
0
20
40
60
80
100
120
140
160
180
Culture Time(hours)

Fig 2

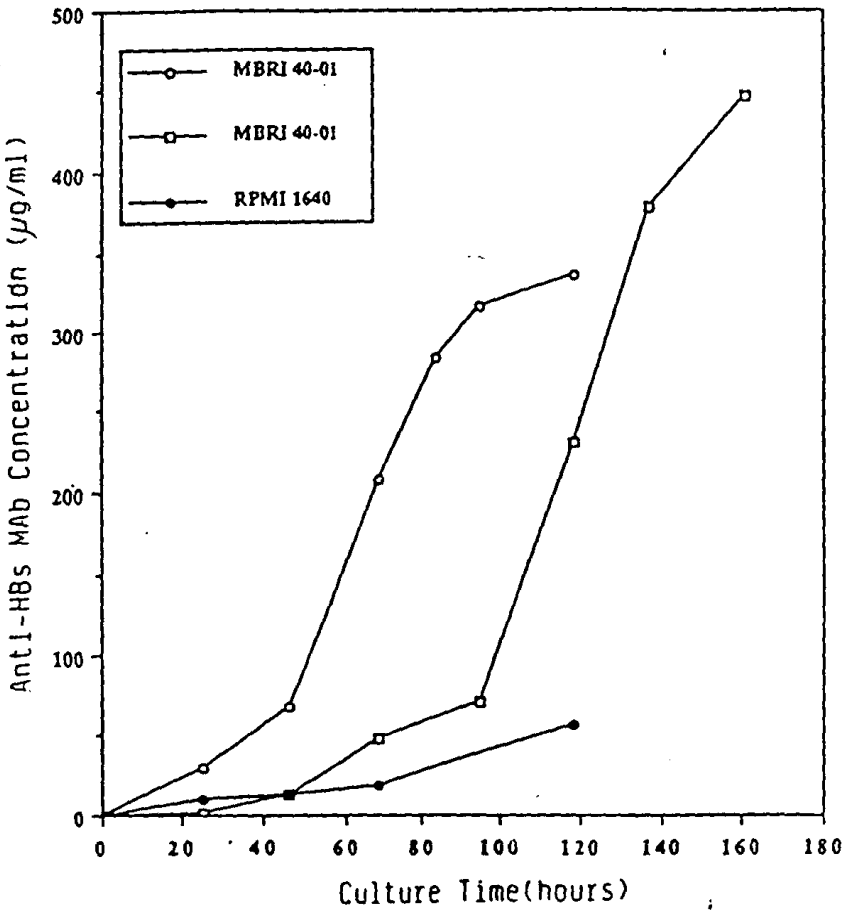

MBRI 40-01
MBRI 40-01
RPMI 1640
Anti-HBs MAb Concentration (μg/ml)
Culture Time(hours)
0
100
200
300
400
500
0
20
40
60
80
100
120
140
160
180

Fig 3

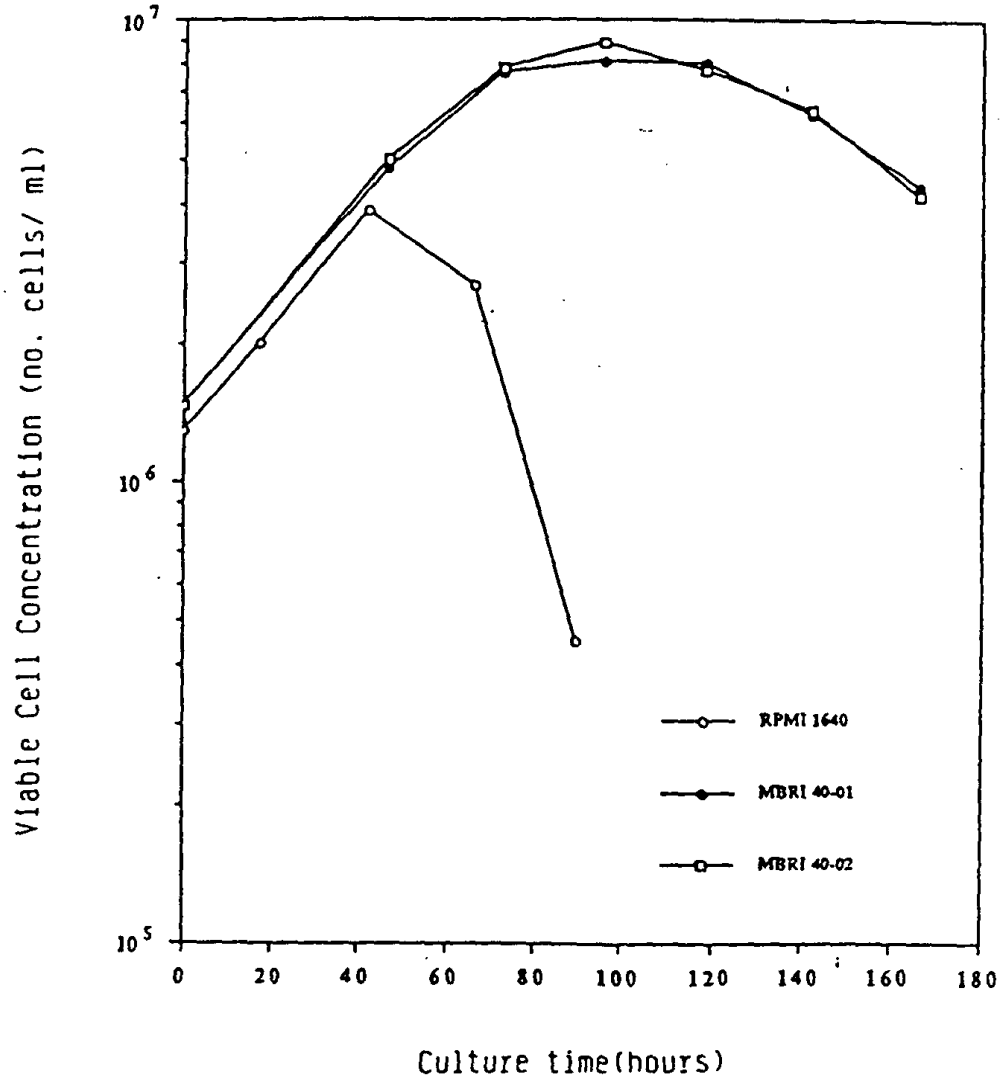

Viable Cell Concentration (no. cells/ ml)
10^7
10^6
10^5
0
20
40
60
80
100
120
140
160
180
Culture time(hours)
RPMI 1640
MBRI 40-01
MBRI 40-02

Fig 4

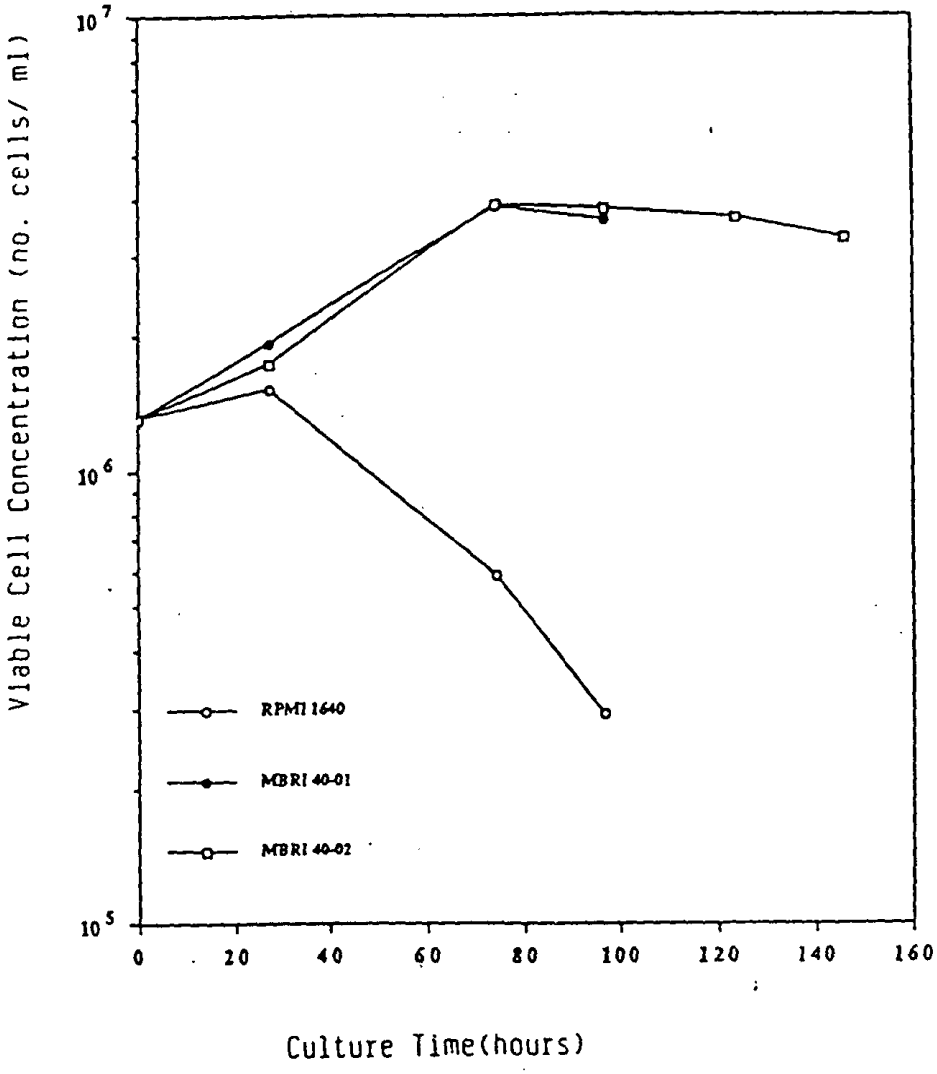

Viable Cell Concentration (no. cells/ ml)
10^7
10^6
10^5
0
20
40
60
80
100
120
140
160
RPMI 1640
MBRI 40-01
MBRI 40-02
Culture Time(hours)

Fig 5

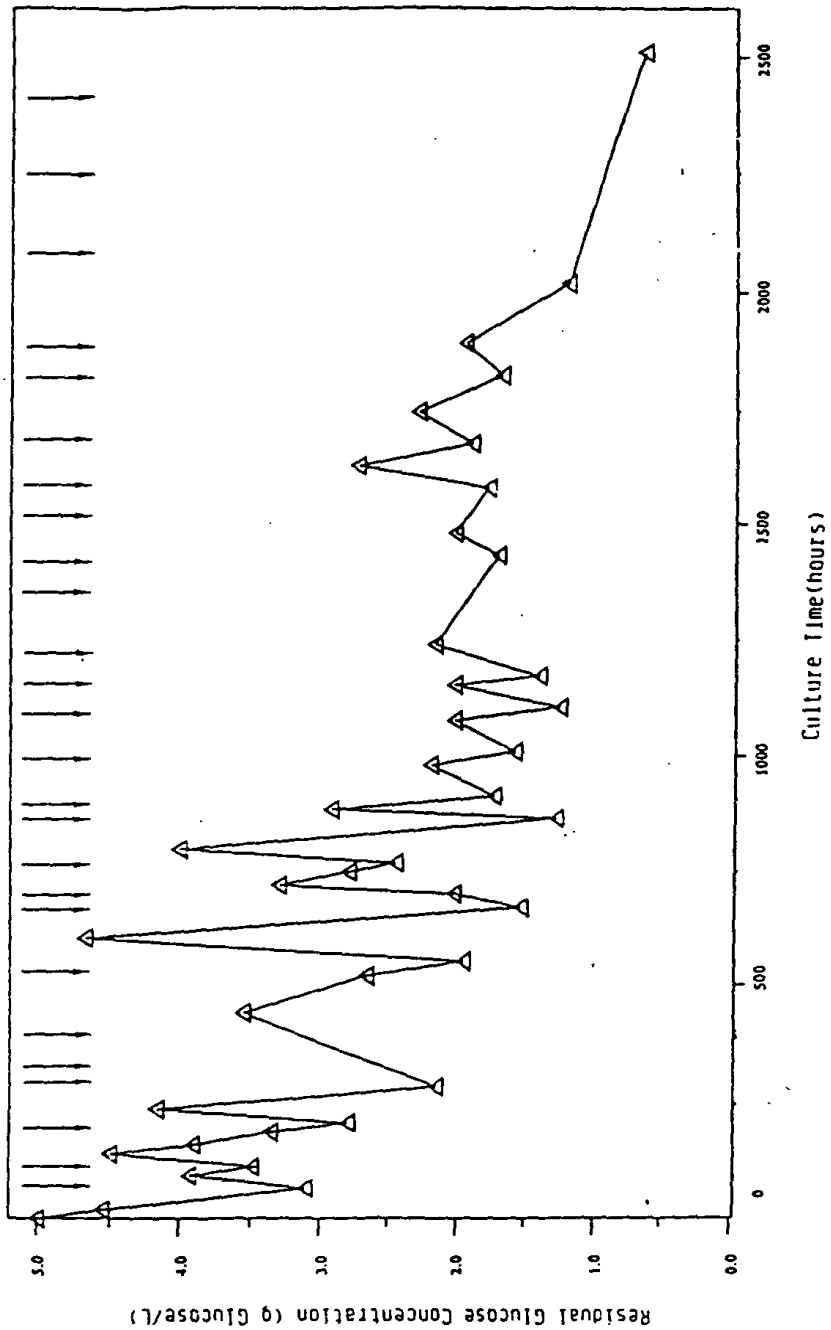

Residual Glucose Concentration (g Glucose/L)
5.0
4.0
3.0
2.0
1.0
0.0
0
500
1000
1500
2000
2500
Culture Time(hours)

Fig 6

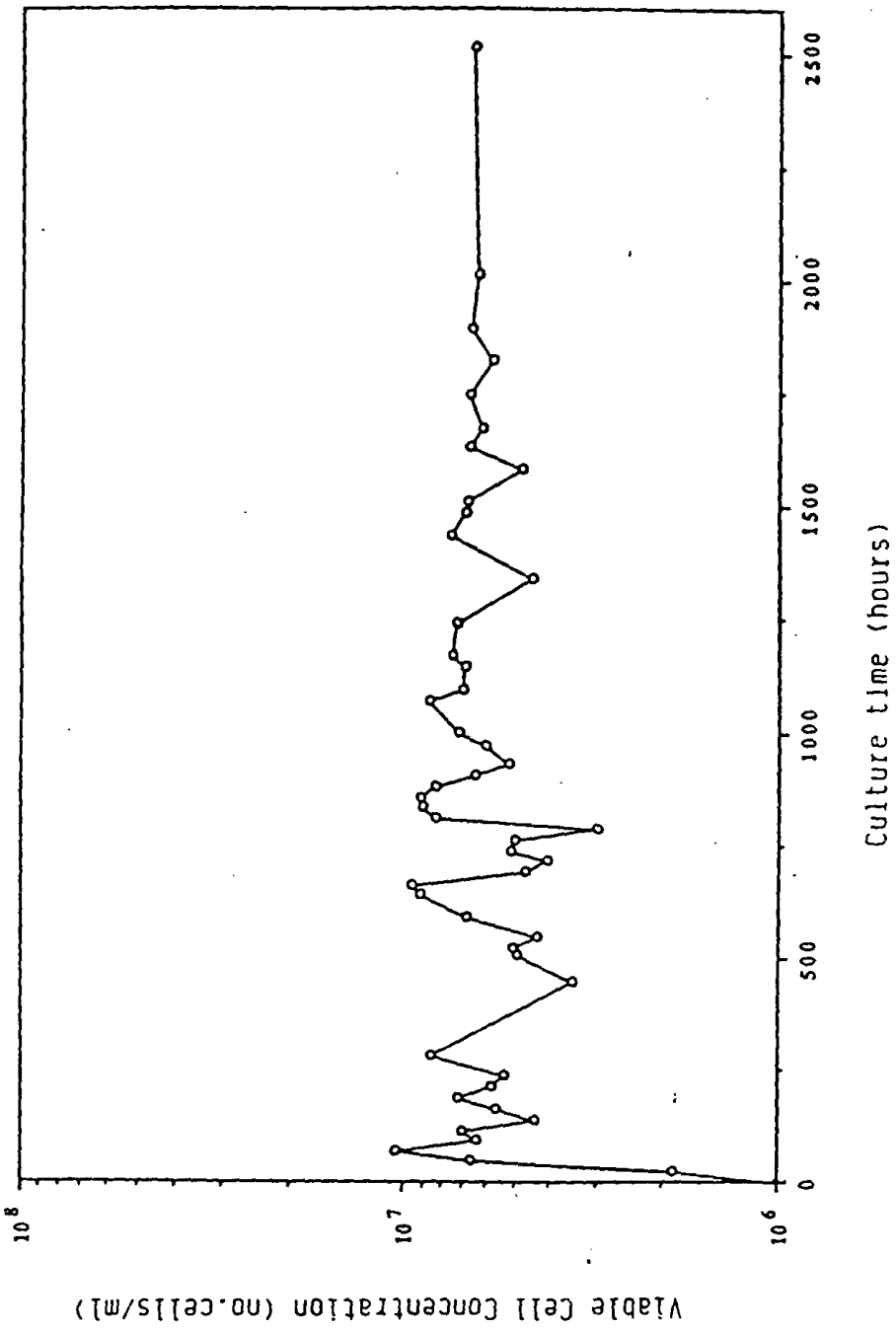
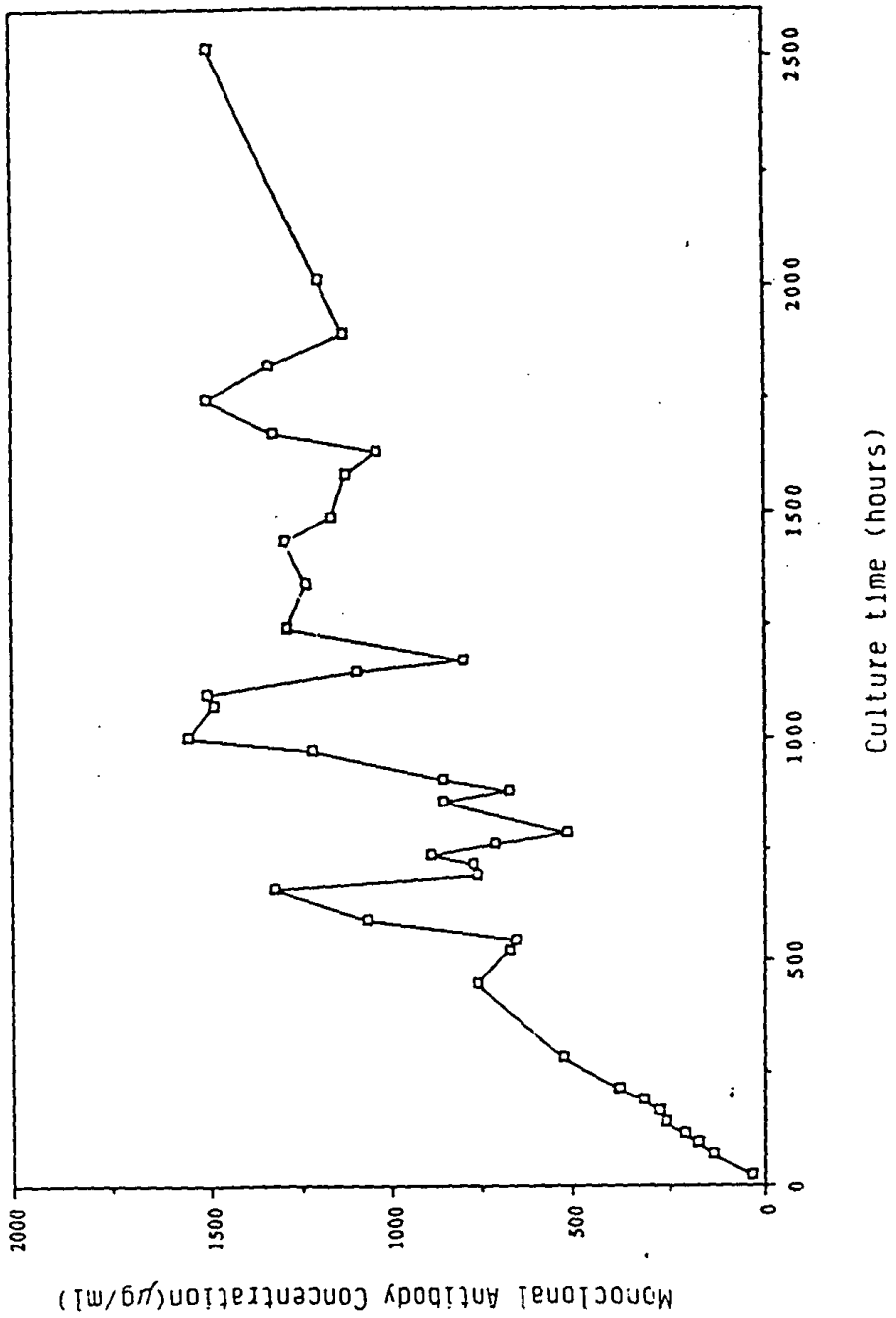

Viable cell concentration (no.cells/ml)
10^8
10^7
10^6
0
500
1000
1500
2000
2500
Culture time (hours)

Fig 7


Monoclonal Antibody Concentration(μg/ml)
2000
1500
1000
500
0
0
500
1000
1500
2000
2500
Culture time (hours)

HIGH-DENSITY MEDIUM FOR ANIMAL CELL CULTURE

This invention relates to high-density medium for animal cell culture and to cell culturing.

In recent years, the advent of various cell culture techniques mostly relying on the application of immobilization methods has enabled in vitro high-density mammalian cell culture to be practicable (Merten, O. W. Concentrating Mammalian Cells. I. Large Scale Animal Cell Culture. Trends Biotechnol. 5, 230-237 (1987); Altshuler, G. L. et al. Continuous Hybridoma Growth and Monoclonal Antibody Production in Hollow Fiber Reactors-Separators. Biotechnol. Bioeng. 28, 646-658 (1986); Jarvis, A. P. et al. Cell Growth and Hemoglobin Synthesis in Murine Erythroleukemic Cells Propagated in High Density Microcapsule Culture. In Vitro 22(10), 589-596 (1986); Velez, D. et al. Comparison of Cell Propagation Methods for Their Effect on Monoclonal Antibody Yield in Fermenters. J. Immunol. Methods 86, 61-69 (1986); Shirai, Y. et al. Continuous Production of Monoclonal Antibody with Immobilized Hybridoma Cells in an Expanded bed fermenter. Appl. Microbiol. Biotechnol. 26, 495-499 (1987); Konrad, W. G. & Hubertus, A. Träger für die Kultivierung

von Menschlichen und/oder Tierischen Zellen in einem Fermenter. European Patent EP 0 205 790 (Dec. 30. 1986); Putnam, J. E. Monoclonal Antibody Production in a Ceramic Matrix. In: Commercial Production of Monoclonal Antibodies, Seaver, S. S. (Ed.). Marcel Dekker, New York, pp. 119-138 (1986)).

However, these techniques have been specifically designed to circumvent the underlying complex problems of cells in vitro. Moreover, these techniques fundamentally have a few problems of fluid dynamics. On the other hand, suspension culture method offers no such problems relatively and has many fundamental advantages for the industrialization of mammalian cell culture system. In addition, it may be a better method to clearly contrast a few problems in realizing in vivo cellular characteristics in vitro and also be a better tool solving those problems (Dodge, T. C. et al. Loss of Viability in Hybridoma Cell Culture - A Kinetic Study. Enzyme Microb. Technol. 9, 607-611 (1987); Backer, M. P. et al. Large-Scale Production of Monoclonal Antibodies in Suspension Culture. Biotechnol. Bioeng. 32, 993-1000 (1988)).

Many previous experiments have been carried out with an expectation that the combined supplement of energy sources, amino acids, and vitamins should lead to an increase in maximum viable cell density over the critical concentration, that is, around (1-3) x 10^6 cells/ml in batch culture (Birch, J. R. & Boraston, R. C. Animal Cell Culture. PCT Patent WO 87/00195 (Jan. 15, 1987); Luan, Y. T. et al. Strategies to Extend Longevity of Hybridomas in Culture and Promote Yield of Monoclonal Antibodies.

Biotechnol. Lett. 9(10), 691-696 (1987); Howarth, W. et al. Cell Culture Medium for Enhanced Cell Growth, Culture Longevity and Product Expression. PCT Patent WO 90/03430 (Apr. 5, 1990)). Nevertheless, those tries have succeeded just in prolonging the stationary phase of the hybridoma growth, which led to a relative increase in overall product yield. The disaccord with the initial expectation has been concluded rashly to be unreasonable without any scientific explanation. However, considering the nutritional balance maintained in vivo, those methods seem to disregard the balance and several important basic conditions for cellular propagation in vitro, and should be in need of nutrient-fortified medium suitable for high-density culture.

In principle, complex interactions occur among components that meet the individual growth requirements of any given type of cell. Under conditions of suboptimal growth, the rate of cellular multiplication is determined by a first-limiting factor(Ham, R. G. Formulation of Basal Nutrient Media. In: Cell Culture Methods for Molecular and Cell Biology. Vol. 1. Barnes, D. W., Sirbasku, D. A. & Sato, G. H. (Eds.) Alan R. Liss Inc., New York, pp. 3-21 (1984)). Therefore, we believe that the total nutritional consideration should be preceded to realize the in vivo cellular characteristics in vitro. However, this might be oversimplified if we just rely on a few nutrients or on empirical rules trying various combinations of nutrient supplement. In conclusion, it is critical to balance all the components in constructing a high-density culture medium.

In this invention, we have disclosed strong evidences that mammalian cells with typical characteristics of cellular growth

can be cultivated up to near 1 x 10^7 cells/ml by fortifying all the components of a conventional cell culture medium. Our experimental examples, which are described in detail in Preferred Embodiments of the Invention, prove that an up-to-date formula of cell culture medium could be re-formulated and further fortified balancedly for high-density culture upon the recognition of the ineffectiveness of nutrient utilization in vitro. Also, the media fortification may enable us to study physiological aspects of mammalian cells in vivo by simulating a real system. For example, the doubtful effect of so-called inhibitory metabolites, such as lactic acid and ammonium ion, should be rescrutinized with the application of this real system. Our experimental results reveal that the major conclusive factor that prevents cells from propagating over the critical density is not those inhibitory metabolites, because the critical cell density is overcome by the balancedly fortified nutrient supply.

The suspension culture method offers many fundamental advantages in scale-up. Further studies and application of this nutrient fortification method in scale-up with the advantages of suspension culture method would create a simple and improved way to the industrialization of the high-density suspension culture system.

This invention relates to the development of balancedly-fortified high-density media for animal cell culture and to the

achievement of a high-density suspension culture using said media.

Namely, this invention relates to the construction of a series of high-density media(MBRI 40-01, 40-02 and 40-03) in which all the nutrient components of the conventional RPMI 1640 medium except inorganic salts were fortified as summarized in Table 1, and to the high-density suspension cell culture processes using said media.

This invention confers higher values to the productions of various pharmaceuticals and biologically active substances produced via animal cell culture by enabling high-density cell culture using said media.

This invention is advantageous to the minimization of the problems encountered during the scale-up for the industrialization of processes by enabling simplification and reduction of conventional culture processes and purification steps thereof.

This invention differs from the conventional high-density culture processes such as perfusion culture, in that our invention only employs the series of said high-density media and, therefore, high-density cell culture is possible without any other equipments specially designed for the high-density cell culture. Consequently, remarkable reduction of investments and maintenance costs for special equipments is plausible.

Figure 1 is growth profiles of hybridoma 2c3.1 in RPMI 1640 medium and in the nutrient-fortified MBRI 40-01 & 40-02 media.

Figure 2 is production profiles of anti-HBs monoclonal antibody produced by hybridoma 2c3.1 in RPMI 1640 medium and in the nutrient-fortified MBRI 40-01 media.

Figure 3 shows growth profiles of human acute lymphoblastic leukemia CCRF-CEM in RPMI 1640 medium and in the nutrient-fortified MBRI 40-01 & 40-02 media.

Figure 4 shows growth profiles of human myeloma RPMI 8226 in RPMI 1640 medium and in the nutrient-fortified MBRI 40-01 & 40-02 media.

Figure 5 shows the residual glucose concentration profile during the fed-batch culture of hybridoma 2c3.1 employing MBRI 40-02 & 40-03 media and feeding scheme of the feeding medium thereby.

Figure 6 is a growth profile of Hybridoma 2c3.1 during the fed-batch culture employing MBRI 40-02 & 40-03 media.

Figure 7 is a production profile of anti-HBs produced during the fed-batch culture of hybridoma 2c3.1 employing MBRI 40-02 & 40-03 media.

In the first step toward the balanced fortification of RPMI 1640 medium for high-density hybridoma culture, we adopted the original molar proportions of each component as such, for the medium had been already developed through procedures of nutrient optimization(Ham, R.G. Formulation of Basal Nutrient Media. In:

Cell culture Methods for Molecular and Cell Biology. Vol. 1. Barnes, D. W., Sirbasku, D. A. & Sato, G. H. (Eds.) Alan R. Liss Inc., New York, pp. 3-21 (1984); Moore, G. E. et al. Culture of Normal Human Leukocytes. J. Amer. Med. Assoc. 199, 519-524 (1967)).

In addition to the molar proportions of each component, several other aspects have been considered to be important. Those are the concentrations of the respective nutrients in serum, inhibitory concentrations of some components against cellular growth, molar ratios of closely-related components and inorganic salts, and the osmolality in the constructed final medium, etc.(Schwartz, W. B. et al. Part XX. Diseases of Metabolism. In: Textbook of Medicine. Beeson, P. B., McDermott, W. & Wyngaarden, J. B. (Eds.). W. B. Saunders, Philadelphia, pp. 1949-1997 (1979)). Combining all those aspects for fortifying medium let the final formulations be specified as described in Table 1. In summary, the RPMI 1640 medium was fortified five times in amino acids, vitamins, and glutathione concentrations, and two and a half times in glucose concentration in MBRI 40-01 medium. The concentrations of inorganic salts were not changed at all except sodium chloride (reduced form 6 g/l to 4 g/l). Therefore, resulting final osmolality was controlled at 270 mOs/kg water. All the same nutrient components in RPMI 1640 medium was fortified ten times in MBRI 40-02 medium, while the concentrations of glucose, glutamine and inorganic salts were as such in MBRI 40-01 medium. MBRI 40-03 medium was designed to be used for a feeding medium in fed-batch culture. It was composed

Table 1. Compositional comparison of the high-density media with RPMI 1640 medium.

mg/L

COMPONENTS	RPMI 1640	MBRI 40-01	MBRI 40-02	MBRI 40-03
INORGANIC SALTS:				
$Ca(NO_3)\cdot 4H_2O$	100	100	100	100
KCl	400	400	400	400
$MgSO_4\cdot 7H_2O$	100	100	100	100
NaCl	6000	4000	4000	- below 2 s/L
$NaHCO_3$	2000	2000	2000	-
Na_2HPO_4	800	800	800	800
OTHER COMPONENTS:				
Glutathione(reduced)	1	5	10	50
Phenol Red	5	5	5	5
D-Glucose	2000	5000	5000	- p.14 2,5g/L
AMINO ACIDS:				
L-Arginine(free base)	200	1000	2000	10000
L-Asparagine	50	250	500	2500
L-Aspartic acid	20	100	200	1000
L-Cystine	50	250	500	2500
L-Glutamic acid	20	100	200	1000
L-Glutamine	300	1500	1500	-
L-Glycine	10	50	100	500
L-Histidine(free base)	15	75	150	750
L-Hydroxyproline	20	100	200	1000
L-Isoleucine(allo free)	50	250	500	2500
L-Leucine(methionine free)	50	250	500	2500
L-Lysine HCl	40	200	400	2000
L-Methionine	15	75	150	750
L-Phenylalanine	15	75	150	750
L-Proline (hydroxy-L-proline free)	20	100	200	1000
L-Serine	30	150	300	1500
L-Threonine (allo free)	20	100	200	1000
L-Tryptophan	5	25	50	100
L-Tyrosine	20	100	200	1000
L-Valine	20	100	200	1000
VITAMINS:				
Biotin	0.20	1.00	2.00	10.0
D-Ca pantothenate	0.25	1.25	2.50	12.5
Choline chloride	3.00	15.00	30.00	150.0
Folic acid	1.00	5.00	10.00	50.0
i-Inositol	35.00	175.00	350.00	1750.0
Nicotinamide	1.00	5.00	10.00	50.0
para-Aminobenzoic acid	1.00	1.00	5.00	50.0
Pyridoxine HCl	1.00	5.00	10.00	50.0
Riboflavine	0.20	1.00	2.00	10.0
Thiamine HCl	1.00	5.00	10.00	50.0
Vitamine B_{12}	0.005	0.025	0.050	0.250

of nutrient components of RPMI 1640 medium f rtified fifty times, while the concentrations of inorganic salts were as such in RPMI 1640 medium. By reducing the concentrations of NaCl (from 6 g/l to 4 g/l for MBRI 40-01 & 40-02 media and to below 2 g/l for MBRI 40-03 medium), the osmolaties of all the high-density media were controlled between 270-320 mOs/kg.water.

High-density culture of a wide variety of anchorage-independent cells can be possible with the application of the series of high-density culture media of this invention. In general, any anchorage-independent cells can be studied for high density culture. Thus, the invention can be used to enhance cell growth and biochemicals production for any specific anchorage-independent cells.

The invention can be further illustrated by the following examples.

EXAMPLE 1

The hybridoma 2c3.1 was cloned by the fusion of CRL 1580 myeloma cells (P3 63.Ag.8.653), to the spleen cells of immunized Balb c mouse with purified human HBsAg (200mg/ml, product of Korea Green Cross). The hybridoma cell line had been deposited to Korean Culture Center of Microorganism (KCCM) according to the Budapest Treaty and designated as KCCM-10003. The hybridoma cells were grown in RPMI 1640 medium supplemented with 10% FBS at 37 °C in a 5% CO_2 in air atmosphere, which stably secreted anti-HBs monoclonal antibody of IgG 1 subtype. Seed-lot system was adopted for spinner flask culture each time in order to maintain the cell

line stably.

Quantitative estimation of the residual glucose concentration was made using a YSI glucose analyzer.

The produced monoclonal antibody was quantitated by an ELISA method of which the standard protocol is as follows; the standard anti-HBs was incubated in HBsAg-coated polystyrene strips at 37°C for 2 hours. Affinity-purified standard anti-HBs was diluted with 0.15M PBS (pH 7.2) containing 5% bovine serum albumin to prepare standard solutions from 4.5 to 300 IU/ml. After the incubation, the polystyrene strip was washed three times with 0.1 ml of 0.1% PBST and incubated at 37°C for 1 hour with 100ng of HBsAg-HRP conjugate diluted with 0.1ml of normal human serum. Then it was washed again five times with 0.1% PBST. The chromogenic substrate solution was amounted to 0.1ml/well at room temperature, followed by the addition of 0.1ml of a stopping solution to each well one hour later. Finally, color development in each well was monitored by a plate reader (SLT Lab.) at 405nm. The standard anti-HBs used for the quantitation of the produced MAb was affinity-purified with culture supernatants of hybridoma 2c3.1 (Adachi, H. et al. Sandwich Enzymoimmunoassay of HBsAg antigen. In: Enzyme Immunoassay, Ishikawa, E., Kawai, T. & Miyai, K. (Eds.) IGAKU-SHOIN, Tokyo, pp. 212-216 (1981)).

In 100ml of nutrient-fortified or original RPMI 1640 media, the hybridoma cells were cultivated with inoculum densities of 1 x 10^5 cells/ml and 1 x 10^6 cells/ml in 250ml Bellco spinner flasks, with stirring at 50 rpm on a biological stirrer (Techne) at 37°C in a humidified CO_2 incubator. The medium was supplemented with

10% foetal bovine serum (Hycl ne). The pH was controlled around 7.0 during the culture with intermittent addition of a sterile 0.4 N sodium hydroxide solution. Viability was determined by the dye exclusion method using trypan blue. With MBRI 40-01 medium, a hybridoma cell line 2c3.1 was cultivated successfully up to a concentration of 9.3×10^6 cells/ml initiating from 1×10^6 cells/ml, while cells cultured in the normal RPMI 1640 medium were dying rapidly after merely 22 hours of culture (Figure 1). With MBRI 40-02 medium, the maximum cell density was 1.2×10^7 cells/ml, which was increased by 30% as compared with that in MBRI 40-01 medium. These data contrastively reveal that the balancedly-fortified nutrient media contribute much to the cellular activity to overcome the in vitro cellular growth limitation by providing a suitable environment for these hybridoma cells.

When the initial cell concentration was 1×10^5 cells/ml, which is a usual inoculum density, cells grew up to 8×10^6 cells/ml in MBRI 40-01 medium (Figure 1). The value is also comparable to that with higher inoculum. The results prove that high-density batch culture is capable irrespective of inoculum density.

As a result of the high-density culture achieved with the nutrient-fortified medium, we have acquired 6 to 8 times higher production of the monoclonal antibody (anti-HBs, IgG1; Figure 2). With 1×10^6 cells/ml inoculum, 340ug/ml of anti-HBs was obtained after 118 hours of culture in comparison to 56 ug/ml in normal RPMI 1640 medium. Even higher concentrations of the monoclonal antibody, 450 ug/ml, was produced in the culture with 1×10^5

cells/ml inoculum after 161 h of culture. This significant increase in monoclonal antibody production can be well expected from the fact that the production of monoclonal antibody is linearly related to the viable hybridoma cell density and the longevity of the period that cells remain viable. In this respect, it is certain that the hybridoma 2c3.1 has been supplied with ample kinds and concentrations of nutrients suitable for high-density culture in batch mode, enough to incur a significant increase of anti-HBs monoclonal antibody.

Example 2

The human acute lymphoblastic leukemia cells(CCRF-CEM; ATCC CCL 119) were subcultured in RPMI 1640 medium supplemented with 10% FBS at 37 ^{o}C in a 5% CO_2 in air atmosphere. In 100 ml of RPMI 1640, MBRI 40-01 and MBRI 40-02 media, respectively, the cells were cultivated with inoculum densities of (1.3-1.5) x 10^6 cells/ml in 250 ml Bellco spinner flasks, with stirring at 50 rpm on a biological stirrer(Techne) at 37 ^{o}C in a humidified CO_2 incubator. The medium was supplemented with 10% fetal bovine serum(Hyclone). The pH was controlled around 7.0 during the culture with intermittent addition of a sterile 0.4N sodium hydroxide solution. Viable cell concentration was determined by the dye exclusion method using trypan blue.

With the nutrient-fortified MBRI 40-01 and MBRI 40-02 media, the cells were cultivated successfully up to concentrations of 8.1 x 10^6 cells/ml and 8.9 x 10^6 cells/ml after 96 hours of

culture, while cells cultur d in the normal RPMI 160 medium were dying rapidly after reaching 3.9 x 10^6 cells/ml at 42 hours of culture(Fig. 3). Our experimental results(Fig. 3) contrastively reveal that the balancedly fortified nutrient supply contributes much to the cellular activity to overcome the in vitro cellular growth limitation by providing a suitable environment for these cells. The results prove that high-density bath culture is also capable for the human acute lymphoblastic leukemia cell line.

Example 3

The human myeloma cells(RPMI 8226; ATCC CCL 155) were sub-cultured in RPMI 1640 medium supplemented with 10% FBS at 37 ^{o}C in a 5% CO_2 in air atmosphere. In 100 ml of RPMI 1640, MBRI 40-01 and MBRI 40-02 media, respectively, the cells were cultivated with inoculum densities of 1.3 x 10^6 cells/ml in 250 ml Bellco spinner flasks, with stirring at 50rpm on a biological stirrer(Techne) at 37 ^{o}C in a humidified CO_2 incubator. The medium was supplemented with 10% foetal bovine serum(Hyclone). The pH was controlled around 7.0 during the culture with intermittent addition of a sterile 0.4N sodium hydroxide solution. Viable cell concentration was determined by the dye exclusion method using trypan blue.

With the nutrient-fortified MBRI 40-01 and MBRI 40-02 media, the cells were cultivated successfully up to concentrations of 3.8 x 10^6 cells/ml and 3.9 x 10^6 cells/ml after 76 hours of culture, while cells cultured in the normal RPMI 1640 medium were dying rapidly after reaching 1.3 x 10^6 cells/ml at 28 hours of

culture(Fig. 4) Our experimental results(Fig. 4) contrastively reveal that the balancedly-fortified nutrient supply contributes much to the cellular activity to overcome the in vitro cellular growth limitation by providing a suitable environment for these cells. The results prove that high-density batch culture is also capable for the human myeloma cell line.

EXAMPLE 4

In 100 ml of this fortified or original RPMI 1640 medium, the hybridoma cells were cultivated with inoculum densities of 1 x 10^6 cells/ml in 250ml Bellco spinner flasks, with stirring at 50 rpm on biological stirrer(Techne) at 37 °C in a humidified CO_2 incubator. The medium was supplemented with 10% foetal bovine serum(Hyclone). The pH was controlled around 7.0 during the culture with intermittent addition of a sterile 0.4N sodium hydroxide solution. Viability was determined by the dye exclusion method using trypan blue.

With MBRI 40-02 medium, a fed-batch culture of the hybridoma cell line 2c3.1 was initiated with an inoculum density of 1 x 10^6 cells/ml(Fig. 6). The feeding medium was MBRI 40-03 medium supplemented with glucose, glutamine and 10% FBS. The concentrations of glucose and glutamine were 2.5 g/l and 750 mg/l respectively. Fed-Batch operation was performed depending upon residual glucose concentration during the culture as indicated in Fig. 5. That is, the residual glucose concentration was maintained over 1 g/l by feeding the fed-batch medium. The volume

of the feeding media was about 10% of the working volume at the time when feeding was performed. The viable c ll concentration reached 1.2×10^7 cells/ml at around 70 hours of culture and it was maintained around $(0.7\text{-}1.0) \times 10^7$ cells/ml for over 2,500 hours of culture as illustrated in Fig. 6.

As a result, the monoclonal antibody production was improved remarkably and high production was maintained for about 2,000 hours after reaching 1 g/l of monoclonal production as illustrated in Fig. 7. The maximum concentration of produced MAb was 1.5 g/l. This significant increase in monoclonal antibody production can be well expected from the fact that the production of monoclonal antibody is linearly related to the viable hybridoma cell density and the longevity of the period that cells remain viable. In this respect, it is certain that the hybridoma 2c3.1 has been supplied with ample kinds and concentrations of nutrients suitable for high-density culture in fed-batch mode, enough to incur a significant increase of anti-HBs monoclonal antibody.

All the results were summarized in Table 2. Maximum cell concentrations in all cultures increased by 2.1-5.2 times as compared with those in the control culture in RPMI 1640 medium. Monoclonal antibody production was enhanced by a factor of 6-8 than that in the control culture. By performing fed-batch culture of a Hybridoma 2c3.1, cell growth and antibody production were enhanced by 3.0-5.2 times and 27 times, respectively, than those values in the control batch culture. Possibility of long-term operation was confirmed by operating the culture up to 2,500 hours.

Table 2. Comparison of enhancements in cell growth and product yield by high-density culture of various mammalian cells.

No.	Cell Lines	Culture Methods	Media	Culture Time (h)	Cell Growth (x 10^6 cells/ml)		Product Conc. (mg/l)	Ratios (MBRI media/RPMI1640)	
					Inoculum Density	Max. Cell Density		Max. Cell Density	Product Yield
1	Murine Hybridoma 2c3.1	Batch Culture	RPMI 1640	118	1.0	2.3	56	-	-
		"	MBRI 40-01	161	0.1	8.0	450	3.5	6.1
		"	"	118	1.0	9.3	340	4.0	8.0
		"	MBRI 40-02	139	1.0	12	-	5.2	-
		Fed-Batch Culture	MBRI 40-02 & 40-03	2,500	1.0	12	1,500	(3.0~5.2)	27.0
2	Human Acute Lymphoblastic Leukemia (CCRF-CEM)	Batch Culture	RPMI 1640	166	1.3	3.9	-	-	-
		"	MBRI 40-01	"	1.5	8.1	-	2.1	-
		"	MBRI 40-02	"	1.5	8.6	-	2.2	-
3	Human Mycloma (RPMI 8226)	Batch Culture	RPMI 1640	97	1.3	1.5	-	-	-
		"	MBRI 40-01	97	1.3	3.8	-	2.9	-
		"	MBRI 40-02	146	1.3	3.9	-	3.0	-

WHAT WE CLAIM IS:

1. A high-density cell culture medium in which all the nutrient components of a conventional medium were fortified balancedly for the purpose of cultivating anchorage-independent mammalian cells.

2. The cell culture medium as claimed in Claim 1, which was constructed by considering factors such as the molar proportions of each component, the concentrations of the respective nutrients in serum, inhibitory concentrations of some components against cellular growth, molar ratios of closely-related components and inorganic salt, salts, and the osmolality in the constructed final media.

3. The cell culture medium as claimed in Claim 1, in which glucose and glutamine has been fortified 2.5 times and 5 times, respectively, and other nutrient components of the media have been fortified 5 times as compared with those concentrations in RPMI 1640 medium.

4. The cell culture medium as claimed in Claim 1, in which glucose and glutamine has been fortified 2.5 times and 5 times, respectively, and other nutrient components of the media have been fortified 10 times as compared with those concentrations in RPMI 1640 medium.

5. The cell culture medium as claimed in Claim 1, in which glucose and glutamine has been fortified 2.5 times and 5 times, respectively, and other nutrient components of the media have been fortified 50 times as compared with those concentrations in RPMI 1640 medium.

6. The cell culture medium as claimed in any one of Claims 3, 4 or 5, in which final osmolality was maintained between 250-320 mos/kg water by controlling the concentration of sodium chloride without changing the concentrations of other inorganic salts as compared to those in RPMI 1640 medium.

7. The cell culture medium as claimed in Claim 1, which can be utilized for the cultivations of anchorage-independent mammalian cells such as murine Hybridoma, human lymphoblastoid cells, and human myeloma cells.

8. A method of growing cells, comprising contacting said cells with the cell culture medium of Claim 3.

9. A method of growing cells, comprising contacting said cells with the cell culture medium of Claim 4.

10. A method of growing cells, comprising contacting said cells with the cell culture medium of Claim 5.

11. The method as claimed in any one of Claims 8, 9 or 10, wherein said cells are antibody secreting cells.

12. The method of growing cells as claimed in any one of Claims 8, 9 or 10, which is performed in a continuous mode by utilizing combinations of said media to produce biochemicals expressed by mammalian cells continuously.

13. The method of growing cells as claimed in Claim 12, in which the feeding of a combination of said media is decided to maintain the residual glucose concentration of the culture supernatant over 1 g/l.

14. The method of growing cells as claimed in Claim 12, in which the long-term operation is performed for up to 2,500 hours.

15. The method of growing cells as claimed in Claim 12, in which said media is fed at about 10% of a working volume of a culture.

16. Biochemicals produced by cells grown in the medium as claimed in Claim 3.

17. Biochemicals produced by cells grown in the medium as claimed in Claim 4.

18. Biochemicals produced by cells grown in the medium as claimed in Claim 5.

19. A cell culture medium substantially as hereinbefore described with reference to any one of the

Examples.

20. A method of growing cells substantially as hereinbefore described with reference to any one of the Examples.

Patents Act 1977
Examiner's report to the Comptroller under Section 17 (The Search Report)

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Relevant Technical fields

(i) UK Cl (Edition K) C6F (FHC1)

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Search Examiner
C SHERRINGTON

Databases (see over)

(i) UK Patent Office

(ii) ONLINE DATABASE: WPI

Date of Search
3 FEBRUARY 1992

Documents considered relevant following a search in respect of claims 3-20

Category (see over)	Identity of document and relevant passages	Relevant to claim(s)
A	WO 90/03430 A1 (CETUS CORPORATION) especially page 7, line 14 - page 9, line 8; Ex 3	3
A	WO 88/01644 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) whole document	3

Category	Identity of document and relevant passages	Relevant to claim(s)

Categories of documents

X: Document indicating lack of novelty or of inventive step.

Y: Document indicating lack of inventive step if combined with one or more other documents of the same category.

A: Document indicating technological background and/or state of the art.

P: Document published on or after the declared priority date but before the filing date of the present application.

E: Patent document published on or after, but with priority date earlier than, the filing date of the present application.

&: Member of the same patent family, corresponding document.

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